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INJECTION OF RESPERPINE INTO ZEBRAFISH, PREVENTS FISH TO FISH COMMUNICATION OF RADIATION-INDUCED BYSTANDER SIGNALS: CONFIRMATION IN VIVO OF A ROLE FOR SEROTONIN IN THE MECHANISM

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□ Serotonin (5-HT) has been implicated as a potential modulator of the bystander effect in cell cultures. To assess the relevance of serotonin in vivo experiments were done with the zebrafish (*Danio rerio*). This species, when irradiated, transmits bystander signals to non-irradiated fish. The animals were injected with reserpine, an inhibitor of serotonin at a dose of 80mg/kg of body mass. The results show that reserpine treated fish had only 27% of the serotonin in non-treated fish. Skin tissue samples were collected from the fish and assayed for bystander signal production using a reporter bioassay. Reserpine prevented the production and communication of signals between fish. Intracellular calcium flux, identified as a bystander response in the reporter cells confirmed this. Medium harvested from tissues of X-rayed fish and their bystanders, showed an oscillating pattern of calcium flux. Samples from X-rayed fish pretreated with reserpine produced a chaotic pattern of random fluctuations in the reporter cells, while their bystander fish led to increased calcium, but no oscillations. These results suggest that 5-HT is involved in bystander signalling in zebrafish, and by decreasing the amount of available 5-HT the bystander effect can be blocked.

Keywords: radiation, bystander effects, intracellular calcium, serotonin (5-HT), zebrafish

INTRODUCTION

Small signaling molecules are the means of communication for the majority of the organisms on our planet. Serotonin (5-hydroxytryptamine, 5-HT) is a chemical signal that has been well conserved for hundreds of millions of years across many phyla and plays a pivotal role in the daily functions of numerous organisms on Earth (Hannon and Hoyer 2008). It serves as both a neurotransmitter and a hormone and acts throughout the body of higher vertebrates, including the central nervous system, peripheral nervous system, cardiovascular system, endocrine system, immune system, gastrointestinal system, sensory perception, as well as many behaviors including sexual, aggression, appetite, sleep, mood, etc (Nichols and Nichols 2008). In more primitive organisms it can serve

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that these chemical signals can modulate cellular response to low doses of radiation *in vitro* (Mothersill *et al.* 2006; Poon *et al.* 2007).

Ionizing radiation has the ability to cause mutations and is also a carcinogen however direct energy deposition in the cell's nucleus is not always needed. The phenomenon of cells exhibiting radiation induced effects, while not being directly irradiated themselves, is known as the bystander effect. As an emerging field in radiation biology, there are numerous reviews, for example (Morgan 2003; Mothersill and Seymour 2001, 2006; Hei *et al.* 2008), though at this point the signal remains unknown and the mechanism has not been fully defined. The bystander effect is not limited to intercellular communication. Recently the transmission of signals between animals has been documented. Both mice (Surinov *et al.* 2004) and fish (Mothersill *et al.* 2006; Mothersill *et al.* 2007; Mothersill *et al.* 2009) are proven models where one group can be irradiated and placed in proximity to a naïve group, the latter group will display the same or similar biological end points as the former. Examples of end points include: increases in sister chromatid exchange, changes in both protein and gene expression leading to mutations, cell death, micronucleus formation, neoplastic transformation (Hamada *et al.* 2007). Protective effects exist as well, such as growth factor production for cell proliferation (Gerashchenko and Howell 2004), growth inhibitory factors for tumor suppression (Komarova *et al.* 1998), and radio-protective adaptive responses (Iyer and Lehnert 2002) which have been documented as end points. Collectively, these are known as non-targeted effects of ionizing radiation.

Calcium transport is an area of interest when studying the bystander effect. In 2002, Lyng *et al.* showed that when non-irradiated cells were exposed to irradiated cell medium there was a transient increase in intracellular calcium levels. Thus transient spikes in intracellular calcium levels became implicated as one of the early events involved in the bystander effect. Further studies by Lyng *et al.* (2006) involved the exposure of HPV-G cells to irradiated cell culture medium and the study suggested that the depolarization of the mitochondria ion membrane potential results in the release and activation of cytochrome c and caspase 3, enzymes involved in the regulation of apoptosis.

Previously, our group set out to examine neurotransmitters, which also act like small signaling molecules in many invertebrates and lower vertebrates, as potential bystander signals (Poon *et al.* 2007). Effects of 5-HT, L-DOPA, glycine, and nicotine on cell cultures receiving irradiation were investigated and it was discovered that upon irradiation of the cells 5-HT was depleted from the medium and bound to 5HT 3A receptors on the cell surface. Focusing on 5-HT as a possible signal, a link to calcium was discovered because 5HT3A receptors are ion-gated calcium channels (Maricq et al 1991). Upon the addition of irradiated cell culture medium,

intracellular calcium measurements showed large transient spikes within a minute. 5-HT alone at micromolar concentrations also caused a bystander effect and reporter cells showed a similar reduction in the number of colonies that survived when given the medium containing 5HT from either irradiated or non-irradiated cells. Reserpine, a substance that depletes monoamines including 5-HT, blocked the bystander effect and clonogenic survival remained higher than in cultures treated with 5-HT (Poon et al 2007).

The previous study was done using an *in vitro* model, and the goal of this investigation is to see whether 5-HT is a modulator of the bystander effect *in vivo*. This study used zebrafish (*Danio rerio*) which as we have previously shown can, when irradiated, communicate signals to fish swimming with them or placed in water in which they were swimming, up to 6 hrs later (Mothersill *et al.* 2007). The hypothesis being tested in this paper is that irradiated fish will pass the bystander signal to non-irradiated fish, however fish that are injected with reserpine will not pass along the signal due to perturbation of the serotonin regulated calcium channel function.

METHODS

Zebrafish Husbandry

Zebrafish (550 – 650 mg), of mixed sex, were purchased from a local fish retailer (Hamilton, ON) and acclimated over two weeks in 40L of dechlorinated Hamilton city water in a glass aquarium. Water temperature was set to 28°C and was mechanically, chemically, and biologically filtered through an external power filter, before being returned to the aquarium via a "waterfall" inflow. This, together with aeration from a diaphragm type air pump and air-stone diffuser, provided adequate aeration. Feeding was twice daily with a commercially available tropical fish flake diet. Full details on husbandry can be found in Mothersill *et al.* (2007). All fish appeared healthy by visual inspection prior to experimentation.

Reserpine injection, X-Ray Irradiation, Bystander Signal Exposure, Sampling Protocol and Tissue Explant Technique

The irradiation protocol followed that of Mothersill *et al.* (2007). Five zebrafish were placed in 200mL of water, then put into an X-Ray machine (Faxitron X-Ray Corporation cabinet system X-Ray system, Wheeling, IL) set to 112keV for five minutes. This has been shown to deliver an average dose of 0.5Gy at a dose rate of 0.1Gy/min (for calibration details refer to; Mothersill et al, 2006). After irradiation, fish were placed in 1L of clean water in a mesh-partitioned container for two hours. Next, five naïve fish were added to the other side of the container and the two groups simultaneously swam for an additional two hours.

Reserpine was dissolved in 0.35 mM acetic acid to give an injection solution of 8 ng ml^{-1}. The fish were then given an intraperitoneal injection with 5 ml; i.e. a total injection dose of approximately 40 ng of reserpine. Given the size of the fish the injection was administered using an Ultra-fine II short needle insulin syringe (Becton Dickinson, Canada). Control injections of the 0.35 mM acetic acid (solvent control) only were also included. The effects of reserpine on both directly irradiated fish and on the bystander fish was evaluated. Thus reserpine injected and non-injected irradiated fish were paired with both reserpine injected and non-injected bystander fish. All injections were given two hours prior to the irradiation or bystander treatment (i.e. X-Ray, Sham X-Ray, or pairing with irradiated or sham irradiated fish). Additional information on sham irradiation or bystander controls can be found in Mothersill *et al.* (2007). All bystander pairings were for 2h.

All the above treatments were carried out on groups of 5 fish. Thus data referring sham X-rayed and X-rayed fish, with and without a reserpine injection, are derived from n = 10 (i.e. 2 groups of 5), since 1 group of each of these treatments was used for pairing with reserpine injected bystander fish and 1 group of each treatment for pairing with non-injected bystander fish. All data referring to completely untreated fish, acid solvent injected and reserpine injected fish (but no X-ray), and all the bystander fish for all the previously listed treatments, are derived from n = 5 fish.

The fish were then killed by cephalic concussion followed by severing of the head. The caudal fin was removed and served as our skin tissue sample. Fins were cut into three equal sized pieces and plated separately in the center of three T25 flasks (NUNC, Uden, Denmark). 2mL of growth medium was added and the flasks were put in an incubator set to 19°C for 48 hours. Treatment of animals and collection of tissue were in accordance with the guidelines of McMaster University and the procedure covered by McMaster University Animal Utilization Protocol (AUP) 06-12-65.

Medium Transfer, and Clonogenic Assay for Bystander Activity

The HPV-G cell line is derived from human keratinocytes isolated from the human foreskin and immortalized by transfection with a plasmid containing the human papilloma virus (Pirisi *et al.* 1987). The cell line was a gift from Dr. J. DiPaolo of NIH, Bethesda, MD. They have been used as a reporter system for the bystander signal in numerous studies (Lyng *et al.* 2006; Mothersill *et al.* 2006; Mothersill *et al.* 2007; Poon *et al.* 2007). The growth medium used for all experiments and all cells was RPMI-1640 (Gibco, Burlington, ON). This was supplemented with 1 mg/mL hydrocortisone (Sigma-Aldrich, Oakville, ON), penicillin-streptomycin solution providing a final concentration of 100 units per ml peni-

cillin and 100mg/ml streptomycin, and 2mM L-glutamine. Fetal bovine serum that was batch screened for the ability to produce a bystander effect in a positive control assay was added to a final concentration of 10%. Hepes buffer (final concentration 30mM) was added to help maintain pH between 7.1 and 7.3. Except where indicated, all reagents were obtained from Invitrogen, Burlington, Ontario.

Upon completion of the 48-hour incubation period, the explant growth medium was poured off donor flasks and filtered through a 0.22mm sterile filter, to remove any cells or debris. The growth medium was also discarded from the recipient flasks and replaced with the harvested donor medium. Recipient flasks had been pre-plated six hrs earlier with a single cell suspension of 500 cells per flask. After 12-14 days, the flasks were stained with 15% carbol fuchsin (VWR, Bridgeport, NJ) and colonies were counted. A minimum of 50 cells was needed for a group to be classified as a colony (Puck and Marcus 1956). Detailed methods on medium transfer and the clonogenic assay, including endpoint measurements from fish explant samples, can be found in Mothersill *et al.* (2006).

Enzyme-Linked Immunosorbent Assay (ELISA)

A 5-HT ELISA kit (Rocky Mountain Diagnostics, Colorado Springs, CO) was used to quantify the amount of serotonin in medium harvested from irradiated explant samples. Samples and reagents were stored at 4°C and allowed to reach room temperature prior to running the ELISA. This ELISA uses the microtiter format as serotonin is bound to the solid phase of the microtiter plate. After all the processing, the ELISA plate is read at room temperature in a plate reader set to 450 nm. The amount of antibody bound to the solid phase serotonin is inversely proportional to the serotonin concentration of the sample.

Ratiometeric Measurement of Ca^{2+}

The method was adapted from Lyng *et al.* 2002 and is briefly described. The Ca^{2+} sensitive dye, Fura-2 acetoxymethyl (AM) (Sigma-Aldrich, Oakville, ON) was used to quantify intracellular Ca^{2+} in the cytoplasm of HPV-G cells. The florescence for the adherent HPV-G cells was measured over time with an automated Olympus 1X81 microscope using a 40X oil objective and a Fura filter cube with 510nm emission. Photon Technology International ImageMaster® 5.0 software was used to control the hardware and for image acquisition.

HPV-G cells were plated at 500 cells in 2ml volume of medium per 35mm petridish (MatTek, Ashland, MA) and incubated at 37ºC and 5% CO_2 approximately 72 hours before measurements. Cells were then washed with a buffer containing 130mM NaCl, 5mM KCl, 1mM Na_2HPO_4, 1mM $MgCl_2$, and 25mM Hepes (pH 7.4). After the washing the cells, 1mL of 4.2µM Fura-2 was added and the plate was left to incubate for a half

hour. Once the incubation period came to an end, the cells were rinsed again with the buffer, and 300µL of fresh buffer was added to each plate for the Ca^{2+} measurement. Twelve bit images were acquired with the camera binning set to 2*2 and exposure time set to 977msec. A field of view of about 10 to 100 evenly distributed cells was randomly selected for the measurement. Fura-2 was excited at 380nm and 345nm and the ratio images were recorded every 2 sec for 7 min. 100µL of the harvested growth medium from samples, serving as bystander signal or a control, was added about 45 sec after the recording began, the point when the baseline was firmly established. The measurements were made in the dark at room temperature.

Drugs and Chemicals

Reserpine was purchased from Sigma-Aldrich (Oakville, ON). It was prepared as a stock solution by being dissolved in glacial acetic acid (Caledon, Georgetown, ON). It was diluted to the appropriate concentration so a dose of 80mg/kg could be given to the fish with the solvent molarity reduced to 0.35mM. Total volume of the injection was proportionate to 1% of the total body weight of the fish. Fura-2 was purchased from Sigma-Aldrich (Oakville, ON) and dissolved in dimethyl sulfoxide (DMSO); the solvent was also purchased from Sigma-Aldrich (Oakville, ON). A stock solution of 420mM was made with aliquots prepared and stored at -20°C in the dark. The working concentration was made by diluting the aliquot in the buffer used to wash the cells before loading the dye, as described above. A final concentration of 4.2mM was used to take the ratiometric measurements of Ca^{2+}.

Statistical Analysis

Clonogenic data are presented as a mean ± standard deviation, where each treatment had n = 5, unless otherwise stated. Clonogenic survival of the HPV-G reporter cells line was analysed by Analysis of Variance followed by Least Square Difference. A $P < 0.05$ was considered statistically significant.

RESULTS

As illustrated in Figure 1, medium from explants from X-rayed fish results in a decrease in clonogenic survival in the reporter cells to 48.5 ± 4.42% of the untreated control value. Explant medium from the bystander fish also results in a significant reduction in surviving cell colonies, 60.8 ± 5.11%. However medium from explants from acid injected fish (i.e. the reserpine solvent control), bystanders to these acid injected fish, and the various combinations of sham treatment control fish data, are not significantly different from the completely untreated con-

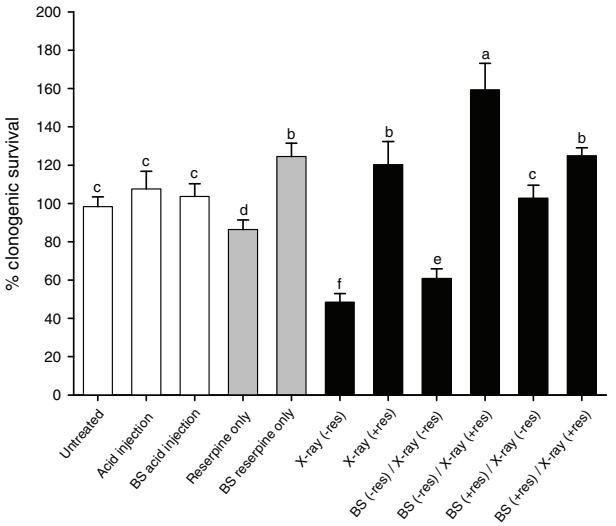

FIGURE 1: Clonogenic survival of HPV-G transfected keratinocyte reporter cells treated with growth medium from zebrafish skin tissue explants. Black bars represent fish that were X-rayed or partnered with X-rayed fish. Grey bars represent treatment or partnership with reserpine only, while the white bars represent untreated and acid injected or partnered control fish. Fish injected with reserpine are indicated by +res and –res indicates those that were not injected. Bars with corresponding letters show statistical similarities. N = 5. BS = bystander fish.

trol fish data (data not shown). Therefore we conclude none of these control treatments had a significant effect on reporter cell clonogenic growth. Explant medium from the reserpine injection alone lowered the reporter survival from 100% to 86.3 ± 5.11%. However when reserpine was administered before an X-ray it negated the effect of the irradiation and the resulting explant medium actually increased reporter survival to 120.3 ± 12.1% of the untreated controls. The bystander partners to this group of fish induced the highest clonogenic survival in the reporter assay, where the clonogenic survival was 159.3 ± 13.8% of the control value.

To confirm that reserpine actually had depleted the level of serotonin in the fish, an ELISA was performed to determine if serotonin was reduced in injected fish. Reserpine injected fish show a decrease in serotonin to 1.76 ± 1.59 ng/mL, from 6.47 ± 3.14 ng/mL in the untreated fish. X-rayed fish had 15.29 ± 11.2 ng/mL, whereas fish pretreated with reserpine and subjected to the same irradiation had 9.10 ± 11.56 ng/mL. Fish injected with reserpine, but not given an X-ray, had only 27% of the 5-HT found in untreated control fish. In the case of irradiated fish, when injected with reserpine before an X-ray, medium from the skin explant had 59% of the 5-HT found in fish that were X-rayed but not injected with reserpine. Reserpine alone did not affect the ELISA showing the serotonin drop occurred in the fish and was not due to the presence of any reserpine in the ELISA.

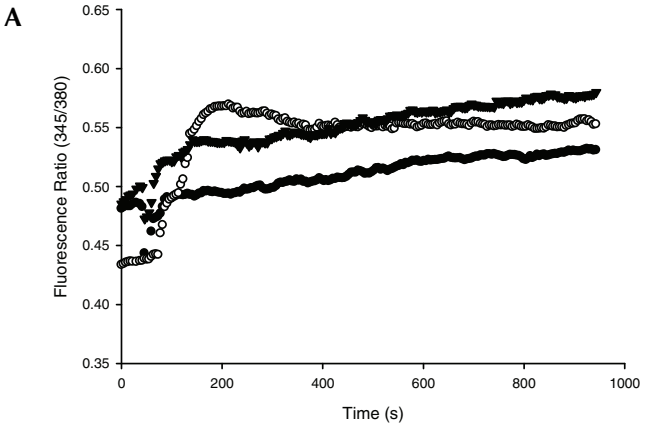

FIGURE 2A: Intracellular calcium trace in HPV-G cells; Sham X-ray fish. Medium was added from fish that were untreated (●), given a Sham X-ray (○), and the bystander fish to the Sham X-ray group (▼), 45 seconds after recording. 6 fields were measured per sample. One representative trace is shown.

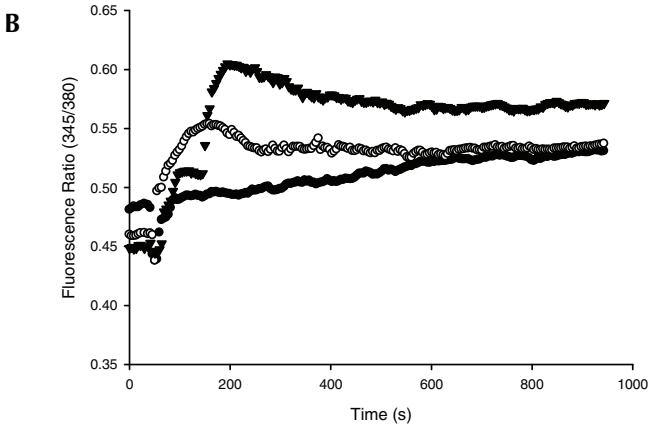

FIGURE 2B: Intracellular calcium trace in HPV-G cells; reserpine injected fish. Medium was added from fish that were untreated (●), injected with reserpine (○), and the bystander fish to the reserpine injected group (▼), 45 seconds after recording. 6 fields were measured per sample. One representative trace is shown.

Figures 2(a-d) show the calcium flux data. Each trace was repeated at least 6 times over 6 randomly chosen fields but because of the difficulty of meaning individual traces, only one representative trace is shown. The medium from untreated fish (2a) caused a relatively small increase in intracellular calcium in the reporters. This pulse has been incorporated into each of the Ca^{2+} measurement traces and acts as a control for comparative purposes. A similar pattern of calcium mobilisation was observed for the bystander fish of the Sham X-ray group. Cells given medium from the Sham X-ray fish also had a similar trace (also shown on Fig 2a).

Figure 2b shows that when medium from reserpine injected fish was applied to reporter cells, a steady increase in intracellular calcium is seen which reached a maximum value of 0.55 before falling to a steady value

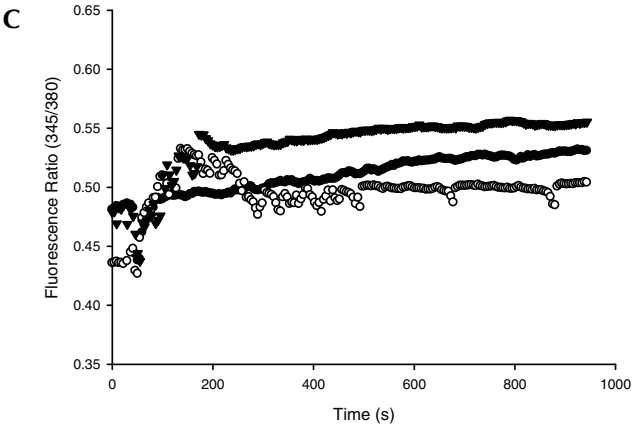

FIGURE 2C: Intracellular calcium trace in HPV-G cells, X-ray fish. Medium was added from fish that were untreated (●), given an X-ray (○), and the bystander fish to the X-ray group (▼), 45 seconds after recording. 6 fields were measured per sample. One representative trace is shown.

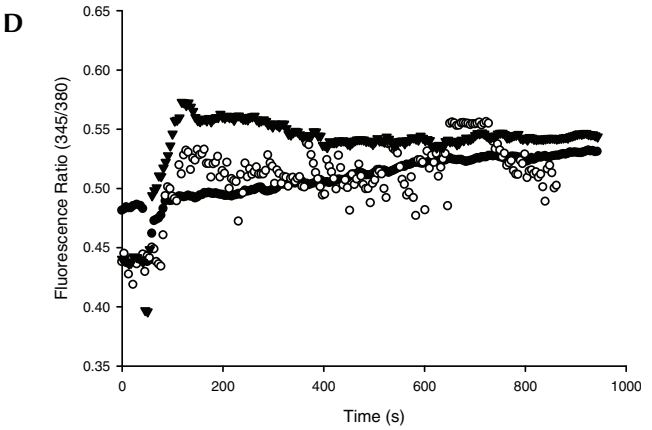

FIGURE 2D: Intracellular calcium trace in HPV-G cells, X-ray + reserpine injected fish. Medium was added from fish that were untreated (●), injected with reserpine and given an X-ray (○), and the bystander fish to the X-ray + reserpine group (▼), 45 seconds after recording. 6 fields were measured per sample. One representative trace is shown.

of 0.53. The medium from the bystander fish of the reserpine injected group also showed a steady increase; though hit a plateau briefly at 0.51 for just under a minute, before rapidly rising past 0.6. After reaching its high of 0.60, it slowly came back down before settling at 0.57.

In Figure 2c the data for reporters receiving medium from X-rayed fish are shown. Here there was an increase in intracellular Ca^{2+}, initially at a rate that was similar to the other traces. However, approximately 175 seconds into the trace frequent oscillations can be observed. These oscillations occur while the ion concentration within the cell is increasing, peaking at 0.53 at 225 seconds post medium addition. The amount of Ca^{2+} in the cytoplasm then begins to drop and oscillates between 0.48 and

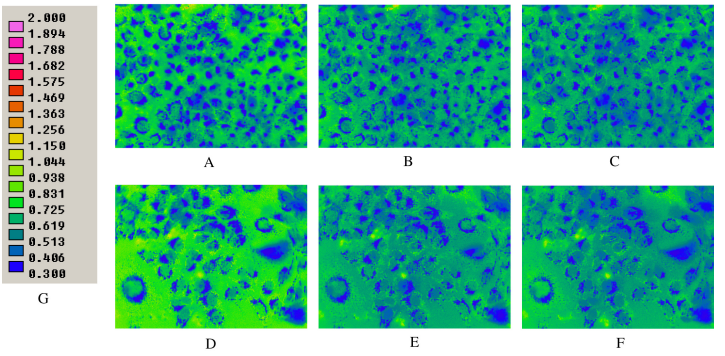

FIGURE 3: Images from ratiometric measurements of intracellular calcium in HPV-G cells exposed to medium from untreated fish and X-rayed fish. Panels A, B, and C represent medium from untreated controls, at baseline (30s), shortly after the addition of medium (90s), and further on after medium addition (150s) respectively. Panels D, E, and F represent medium from X-rayed fish, at baseline (30s), shortly after the addition of medium (90s), and further on after medium addition (150s) respectively. Panel G is a legend indicating the ratio of fluorescence (345nm:380nm) represented by each colour.

0.51. This decrease lasts for a further 200 seconds, before it settles around 0.49 with minor oscillations during the remainder of the measurement. The trace for the bystander fish to the X-ray group, shows several oscillations as the concentration of Ca^{2+} rises after addition of the medium. After briefly reaching a plateau of 0.53, it dips back down to 0.51. 175 seconds later it shows a rapid escalation to 0.55 before coming back down slightly to between 0.53 and 0.54 for 25 seconds, followed by a slow increase to about 0.55 for the rest of the measurement.

Random and frequent oscillations of Ca^{2+} can be observed in the HPV-G cells upon adding medium from fish that were given a reserpine injection followed by an X-ray (Figure 2d). There is a clear rise that reaches a maximum of 0.53, but after there is no clear pattern. There are random spikes, as high as 0.54, as well as crashes, to as low as 0.47 in the ratio. The accompanying bystander fish show a large increase as well reaching a maximum ratio of 0.57 at 175 seconds. After this time the ratio begins to decrease slowly to 0.54, where it remains with a few small oscillations.

One final observation from this set of experiments is that the medium from all of the bystander fish finished with a higher ratio than the medium from their counterparts be they Sham X-ray, X-ray, reserpine injected, or a combination of reserpine injected and X-ray.

Images taken during fluorescence measurements are shown in Figure 3. As the ratio increases (colour changes from blue towards green), calcium concentration is rising, as the ratio decreases (colour changes from green towards blue) calcium concentration is falling. Panels A through C show the concentration changes in cells which received medium from untreated control fish. Calcium rises slightly within the cell, and has a moderate drop on the extracellular area surrounding cells. As the meas-

urement proceeds, there is very little change in the ratio. Panels D through F show the concentration changes in cells receiving medium from the X-rayed fish. There is a higher increase in calcium compared to the controls and fluctuations in the amount of calcium inside the cells as the measurement continues.

DISCUSSION

The primary hypothesis of this study was that 5-HT has a role in modulating the bystander effect in vivo, and the data from our experiments support this idea.

The degree of variation of 5-HT in the medium of all the groups is high. This is probably because 5-HT is normally highly variable in vivo as it is involved in regulating a wide selection of functions. For example, Lepage *et al.* (2005) report that by increasing 5-HT in rainbow trout, aggression and hostility seen in dominant fish is suppressed. Zebrafish are not known to be aggressive fish, however they do display aggressive interactions that are slightly more subtle making them harder to notice (Larson *et al.* 2006). It is likely that some of the zebrafish were dominant, whereas others were subordinate, thus causing a natural variability in 5-HT.

The results from the reporter bioassay show that reserpine does successfully block the bystander effect in the zebrafish. This leads to a higher level of colony growth in reporter cells which often exceeded the control value. Fish injected with the acetic acid solvent only, show similar values to the untreated controls, as do the bystander fish that were paired with these control groups. The data from the various controls show that the reduction in survival in the reporter cells is only occurring in the irradiated group and the bystander partner fish.

The reporter assay identifies late effects of irradiation or signal production therefore the calcium ratiometric assay was used to identify early events in the process. Since serotonin is thought to act by opening calcium type 3a channels (Poon *et al.* 2007), the final stage of this study involved measuring intracellular calcium. Medium from the untreated control fish did not show a significant change in intracellular calcium. Medium harvested from explants of both reserpine injected fish and the bystander fish medium produced an increase in the intracellular concentration of calcium however the effect was not as strong as that seen in x-rayed fish. Oscillations in calcium levels are seen in reporter cells exposed to medium samples from X-rayed fish and their bystanders - the former showing oscillations for 500 seconds, while the latter show oscillations only during the first 150 seconds. Even more interesting is the unusual chaotic oscillations produced by samples from X-rayed fish pretreated with reserpine. The bystander fish to this treatment shows no unusual pattern suggesting that the reserpine injected fish did not communicate signals to the bystander fish. It is important to point out that measurements

of intracellular calcium fluxes are highly variable, and meaning several individual traces usually masks effects. Therefore the pattern of the trace is the significant endpoint and in these experiments each of six replicate cultures in which 6 random fields were recorded showed the patterns reported.

To analyse the significance of these traces requires an understanding of the role of calcium in membrane signaling. The increase in calcium is from extracellular sources (Lyng *et al.* 2006), and not from intracellular reserves like the endoplasmic reticulum or mitochondria. As the influx of calcium ions increases, it likely happens at a rate that would normally be toxic to cells. The mitochondria are able to act like a calcium sink (Ganitkevich 2003), when there is an over abundance of calcium. Typically the majority of the ions entering the mitochondria are bound, while a small percentage is free. However, if the concentration exceeds a threshold where calcium-binding proteins become saturated and the buffer within the mitochondria is unable to control the amount of free calcium in the matrix, the permeability transition pore, a channel within the mitochondria opens leading to cell death (Hajnoczky *et al.* 2001). Also, as free calcium ions in the mitochondria increase, ATP production increases, generating more reactive oxygen species (ROS). An increase in ROS raises the likelihood of cell death and has been linked by many workers to genomic instability and bystander effects (Lyng *et al.* 2002; Dedkova and Blatter 2008; Shao *et al.* 2008).

The fluctuations in calcium, depicted by the oscillations, could mean several things. Constant oscillations are a disruption to homeostasis for both the cytoplasm and mitochondria. Kaftan *et al.* (2000) describe the effects of rapid alternations between high and low concentrations of calcium in gonadotrope cells. Binding of gonadotropin-releasing hormone on the receptors of the cell, causes intracellular calcium oscillations. These fluctuations induce the secretion of luteinizing hormone and follicle stimulating hormone. Perhaps a similar mechanism is at work in irradiated cells, where oscillations of calcium trigger the secretion and release of the bystander signal. Furthermore, in the gonadotrope cells there is a higher concentration of calcium at local sites where the secretion actually takes place. This could be the case when looking the image of the HPV-G cells during the calcium measurement; from Figure 3 one can see various shades of colour, translating to different concentrations of calcium. In the cells given X-ray medium, there is a continual change in local concentrations. This raises the possibility that the variations in calcium concentration within a cell may be a sign that secretion of the bystander signal is taking place. The chaotic pattern seen when reserpine is used would suggest interference with the secretory mechanism. Next, since the mitochondria are a calcium ion sink, as the intracellular concentration of calcium increases, there is likely a time lag where the

organelle takes in the ions. This would correlate to the increase aspect of the oscillation as more ions are entering the cytoplasm than there are entering the mitochondria. As the mitochondria catch up and accumulate calcium, the decrease phase of the oscillations may occur. As the ions enter the organelle, eventually there may be another delay as the binding proteins yet to take calcium become fewer, which would lead to another rise in calcium concentration. A cycle like this could disrupt normal function and lead to cellular malfunction.

It would also be interesting to see if there is a difference based on the type of antagonist used. Reserpine blocks the transporting ability of VMAT while ondansetron acts directly on the 5-HT_3. In both cases, the antagonist prevents 5-HT from interacting with the cells and results in 5-HT remaining on the extracellular side, where it is broken down. In conclusion, 5-HT appears to be involved in the bystander effect signal production and transmission both in vitro and in vivo. This suggests it could be a valuable drug for modulating radiation effects in both the clinic and the environment.

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